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Two-Centre Study Comparing DNA Preparation and PCR Amplification Protocols for Herpes Simplex Virus Detection in Cerebrospinal Fluids of Patients With Suspected Herpes Simplex Encephalitis

H.H. Hirsch[1] and W. Bossart[2]*

[1]*Institute of Medical Microbiology, University of Basel, Basel, Switzerland*
[2]*Institute of Medical Virology, University of Zurich, Zurich, Switzerland*

In a two-centre study, the routine DNA preparation and PCR amplification protocols were compared for herpes simplex virus (HSV) detection in cerebrospinal fluids (CSFs) of 43 patients with suspected herpes simplex encephalitis (HSE). The combined clinical, radiological and laboratory results indicated HSE in 6/43 (14%) patients. Discrepant PCR results between the two centres were obtained in 8 (18%) cases consisting of 5 false-positive and 3 false-negative results. Seven out of 8 (88%) discrepant results were associated with the method of CSF preparation using protease K digestion followed by heat inactivation. In contrast, CSF digestion with proteinase K followed by DNA purification on silica spin columns was better yielding discrepant PCR results in only 1 of 78 analyses (1.3%). The results point to the need for standardization and inter-laboratory quality control for routine clinical work. ***J. Med. Virol. 57:31–35, 1999.***

KEY WORDS: neurologic disorders; central nervous system; neurotropic viruses

INTRODUCTION

The polymerase chain reaction (PCR) on cerebrospinal fluid (CSF) has been suggested as the method of choice for diagnosing herpes simplex virus encephalitis (HSE) [Lakemann et al., 1995; Cinque et al., 1996; Mitchell et al., 1997]. Thus, molecular virology laboratories have acquired a pivotal role in the early diagnosis of HSE. The high mortality of HSE of approximately 80%, the availability of specific antiviral therapy and the fact that CSF sampling is carried out more readily than brain biopsy challenge the diagnostic laboratories with increasing numbers of examinations that have to be undertaken at a high speed and performance level. However, standardization of PCR is lacking and external inter-laboratory quality control is only at the beginning of implementation. For those viruses for which international collaborative studies on standardization of diagnostic PCR had been carried out the results were discouraging in that more than half of the participating centres failed to diagnose all samples correctly [for example see: Pauw et al., 1995; Damen et al., 1996]. Outside of the research setting, therefore, the role of PCR for the diagnosis of HSE may be overestimated [Landrey, 1995]. In an attempt of preliminary inter-laboratory quality control between two Swiss University Centres, we compared the results of routine PCR protocols for herpes simplex virus detection in the CSFs of 43 patients with suspected HSE. Each centre was using a different method for DNA preparation and a different nested PCR amplification protocol. The results confirm the need for standardization and further inter-laboratory quality control.

MATERIALS AND METHODS

The Centres

Centre A provides the majority of all viral PCR diagnostic services for a Swiss University Hospital amounting to more than 2600 PCR analyses during 1996. Centre B provides viral PCR services mainly to the Neurology and the Paediatric Departments of the largest University Hospital in Switzerland. Together, the two medical centres serve approximately one-fifth of the total Swiss population.

Cerebrospinal Fluids (CSFs)

From CSF samples submitted for routine PCR tests to each of the two centres, 43 CSFs from patients with

The current address of Hans H. Hirsch, MD, MS, FAMH is Medical Microbiology, Medical Clinic of the University of Basel, Kantonsspital Bruderholz, CH-4101 Bruderholz BL, Switzerland.

*Correspondence to: Walter Bossart, PhD, Institute of Medical Virology, University of Zurich, Gloriastrasse 30, CH-8028 Zurich, Switzerland. E-mail: BOSSART@IMMV.UNIZH.CH

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suspected HSE were examined (Centre A: 21 consecutive samples of 1995/96; Centre B: 22 selected samples including all samples which had been positive or demanded a repeated work-up in 1995/96). The CSF samples were stored at –20°C before DNA preparation.

Analysis of Case Reports

Case reports were collected from each of the 43 patients in order to qualify HSE as probable or unlikely on the basis of the combined information on clinical course, radiological findings on magnetic resonance imaging (MRI), computed tomography (CT) scans, electroencephalogram (EEG), and/or identification of another aetiology. For 6 patients, the results of MRI and/or CT scans of the brain were compatible with the diagnosis of HSE. In 3 patients, an encephalitis of viral origin could not be excluded, but specific evidence for HSE was lacking, and were assigned as negative for the purpose of this study. In 34 patients, HSE could be ruled out according to the clinical course without acyclovir therapy, or by the demonstration of another cause of disease. Based on these data, PCR results were qualified as true positive, true negative, false positive, and false negative. None of the 6 HSE cases were immunocompromised individuals. Four of the 43 patients (two in each centre) were infected with HIV-1 (CDC classification B1 and C3), all four being negative for HSV in CSF.

DNA Preparation

The methods for DNA preparation and the amplification protocols in the study were those used routinely at each centre for PCR analysis of CSF. Centre A used a proteinase K digestion step followed by purification of the DNA on silica spin columns (QIAmp Blood Kit, product number 29104/6, QIAGEN; preparation procedure according to the manufacturer's instructions version 5/95). Centre B used proteinase K digestion followed by heat inactivation at 94°C for 12 minutes [Greenfield and White, 1993].

PCR Methodology

Both centres used a nested PCR amplification protocol for reasons of increased sensitivity: In Centre A, a nested PCR protocol for the UL42 gene modified after Puchhammer-Stöckl et al. [1990] was applied. The outer primers were M162 (5′-GGCATCCTTATCCATAACACGA-3′) and M163 (5′-GACGACCACAGACAAGGTGCGA-3′), the inner primers M164 (5′-ACGACGACGTCCGACGGCGA-3′) and M165 (5′-GTGCTGGTGCTGGACGACAC-3′) yielding PCR products of 615 bp in the first run and 278 bp in the second run. Deoxy-UTP was substituted for TTP in all amplification reactions. An uracyl-N-glycosylase decontamination step (10 min, 37°C) was carried out before the first round of PCR. Amplification was done in a Perkin Elmer Gene Amp System 9600 thermocycler running 35 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec. Five μl of the first amplification reaction was transferred to 45 μl of pre-pipetted master mix containing the inner primers, but no uracyl-N-glycosylase, and the second amplification was carried out running 40 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec. In addition to diluted HSV-1 culture supernatants, external high- and low-positive controls consisting of 15 and 30 copies of cloned plasmid target DNA in 200 ng of peripheral blood leukocyte DNA and peripheral blood leukocyte DNA as negative control were used. Routinely, 5 amplifications were done per diagnostic patient block consisting of a negative control, a duplicate of the CSF to be analysed, a second negative control, and a target amplification control consisting of the patient CSF preparation spiked with 30 copies of the HSV UL42 target DNA prior to PCR. When the CSF duplicates in one diagnostic block showed discrepant results, the CSF analysis was repeated in quadruplicates. In Centre B, a nested protocol for the glycoprotein D gene according to Aurelius et al. [1991] was used. The outer primers were BJHSV1.1 (5′-ATCACGGTAGCCCGGCCGTGTGACA-3′) and BJHSV1.2 (5′-CATACCGGAACGCACCACACAA-3′), the inner primers BJHSV1.3 (5′-CCATACCGACCACACCGACGA-3′) and BJHSV1.4 (5′-GGTAGTTGGTCGTTCGCGCTGAA-3′), yielding PCR products of 221 bp and 138 bp, respectively. Amplification was done in a Perkin Elmer Gene Amp System 2400 thermocycler running 35 cycles of 94°C for 15 sec, 58°C for 30 sec, 72°C for 30 sec. After transfer of 5 μl of the first round PCR into 45 μl of newly prepared mastermix containing the inner primer pair, the second amplification was performed running 20 cycles of 94°C for 15 sec, 58°C for 30 sec, 72°C for 30 sec. Patient samples were tested routinely in duplicates; test samples again were spaced by negative and positive control reactions. In both centres, PCR products were detected by agarose gel electrophoresis and ethidium bromide staining and identified by comparison with the PCR products of the external positive controls. The nested PCR protocols used at both Centres were specific for HSV DNA and did not yield amplicons of the respective HSV target sizes with human peripheral blood leukocyte DNA or DNA from cell cultures infected with Varicella-Zoster virus, Cytomegalovirus, Epstein-Barr virus, Human Herpesvirus 6. The threshold of detection in Centre A was determined by dilution as 5 genome equivalents and monitored routinely using 15 and 30 copies of cloned HSV amplicons. In Centre B, the HSV yield of infected green monkey kidney cells (Vero) was titrated prior to DNA extraction, and the infectivity of 0.05 infectious units was determined as threshold of detection by PCR.

RESULTS

PCR examination of the 43 cerebrospinal fluids yielded 6 (14%) positive results. Typical gel patterns of positive and negative results for each of the two centres are shown in Figure 1. In 5 out of 6 cases positive results could only be detected after amplification with internal primers, i.e. after the "second run" of the

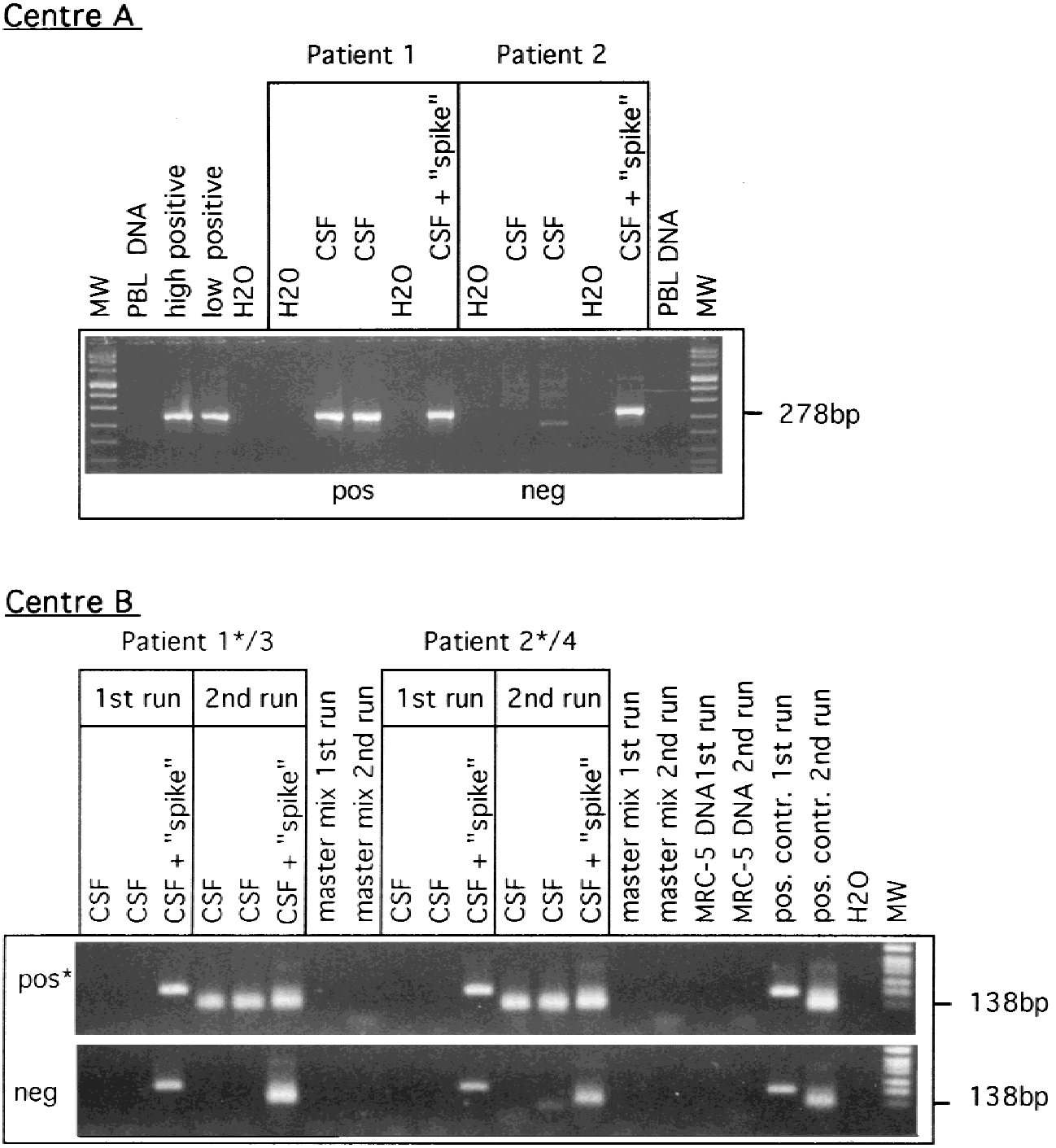

Fig. 1. Gel patterns of PCR analyses of cerebrospinal fluids for Herpes simplex virus type 1 DNA as yielded by each of the two centres. Both centres used a nested PCR amplification protocol for reasons of increased sensitivity. Positive results typically could only be detected after amplification with internal primers, i.e. after the "second run" of the nested PCR amplification protocol (see results of Centre B).

nested PCR amplification protocol (see results of Centre B).

In the first series, 10 CSF samples were processed for PCR by protease K digestion followed by heat inactivation. Centre A identified correctly all 10 samples (1 true positive, 9 true negative results), whereas 2 false positive results were registered at Centre B (see Series I, Table I). Accordingly, a 100% sensitivity and 100% specificity were calculated for the PCR procedure used in Centre A, while 100% sensitivity, but only 78% specificity were obtained by the procedure used at Center B.

The second series of 22 CSF samples was prepared for PCR by proteinase K digestion followed by DNA purification using silica spin columns. The series of

TABLE I. Results of Two-Centre Study Comparing DNA Preparation and PCR Amplification Protocols for Herpes Simplex Virus Type 1 Detection in Cerebrospinal Fluids (CSFs) of Patients with Suspected Herpes Simplex Encephalitis (HSE)

Test Series	DNA preparation method	Centre	Number of CSF tested	PCR: positive	PCR: negative	Discrepant results	Comparison with final diagnosis: TP	TN	FP	FN
I	protease + heat	A	10	1[b]	9	2	1	9	0	0
		B		3	7		1	7	2	0
II	protease + column	A	22	3	19	0	3	19	0	0
		B		3	19		3	19	0	0
III	protease + column	A	17[a]	2	15	6	2	14	0	1[b]
	protease + heat	B		4	13		1	11	3	2
	protease + column	B		3	14	5	3	14	0	0

Center A: Nested PCR protocol for UL42 gene (modified after Puchhammer-Stöckl et al., 1991).
Center B: Nested PCR protocol for glycoprotein D (Aurelius et al., 1991).
TP, true positive; TN, true negative; FP, false positive; FN, false negative.
protease + heat: CSF preparation for PCR using protease K digestion followed by heat inactivation; protease + column: CSF preparation for PCR using protease K digestion followed by DNA purification on silica spin columns.
[a] 6 CSF samples tested in series I are re-prepared and re-tested in series III.
[b] this CSF sample tested TP after protease + heat digestion, but FN after protease digestion + DNA purification on silica spin column

samples were tested at each centre by the respective PCR protocol. Both centres recognised all 22 samples with no discrepant results (3 true positive, 19 true negative results, Series II in Table I) which corresponded to 100% sensitivity and 100% specificity for both PCR protocols. These results suggested the possibility that the sample preparation method based on protease K digestion and heat inactivation may be inferior to a method using a DNA purification step after proteolytic digestion.

Therefore, the third series of 17 CSF samples which included 6 CSF samples of Series I was prepared at one Centre (Centre B) using both DNA preparation protocols in parallel which was then analysed by the same PCR protocol. As shown in Table I, 5 discrepant results were noted, the CSF preparation method of protease K digestion and heat inactivation being associated with 3 false positive and 2 false negative results despite the use of the same routine amplification protocol. The CSF preparations of Series III obtained by spin column purification was also analysed in Centre A, yielding only 1 discrepant result (1 false negative result; Series III in Table I).

DISCUSSION

PCR-based detection of the HSV genome in CSF has been suggested as the emerging "gold standard" for the diagnosis of HSE [Lakeman et al., 1995; Mitchell et al., 1997] whereas the established "gold standard" of brain biopsy is not or only reluctantly being undertaken at most centres [Cinque et al., 1996]. Thus, most published PCR protocols as applied in the routine laboratory are not adequately evaluated except in a few well conducted research settings [Lakeman et al, 1995]. As an alternative, we cross-checked at two Swiss University centres two different routine protocols used for HSV detection in CSF samples of 43 patients and identified 6 patients with HSE (14%), a percentage similar to that found in other studies [Whitley, 1990; Cinque et al. 1996; Koskiniemi et al., 1996]. Three lessons can be learned from this comparison:

1. The pre-PCR processing of the clinical sample in order to render the DNA accessible for amplification is most important for reliable PCR results. In our hands, the simple procedure of lysis followed by heat inactivation of the proteinase K, as it was recommended as sufficient for a comparatively non-complex body fluid such as CSF [Greenfield and White, 1993], was clearly inferior to an improved procedure of proteolysis followed by DNA purification on silica spin columns. The crude lysate preparation may contain potentially inhibitory substances which are removed by the spin column step. However, this procedure was not only associated with false negative, but also with false positive results (Series I+III, Centre B, Table 1). It is suspected that this may result from the heat inactivation step at 94°C during which the sealing of the tubes may not be adequate leading to cross-contamination of samples in the subsequent processing steps. In contrast, protease K digestion and silica gel adsorption of DNA yielded but 1 false negative result at Centre A in a total of combined 78 tests at the two centres. Of note, this sample of series III had been found to be truly positive by Centre A in Series I suggesting the possibility of low HSV DNA copy numbers (below 5 copies) and/or stochastic distribution. Based on the above results, Centre B has switched to DNA purification on silica spin column as routine method of CSF preparation for PCR analysis.

2. Despite the different HSV target genes and amplification protocols used at the two Centres, the HSV UL42 and the glycoprotein D gene regions were found to be equivalent with respect to sensitivity and specificity when the same improved CSF preparation was used (Table 1, Series II and III). In addition, the nested PCR format *per se* was not the reason for false positive results provided proper controls were used. To what extent this finding can be extrapolated to other HSV target gene sequences remains to be evaluated. Our own observation indicates that this holds also true for the HSV polymerase gene using a protocol of single-

round PCR followed by hybridisation and EIA detection (unpublished observation).

3. Even though both centres claim to run diagnostic PCR at a high performance level and take maximal precautions for preventing contamination, our comparison revealed false positive and false negative results. Without improvement of the CSF preparation method, this has a negative impact on the role of PCR for HSE diagnosis and treatment as well as on the search for other aetiologies, not even taking into account rare cases with a second pathogen [Koskiniemi et al., 1996; Tang et al., 1997]. In the absence of PCR quality control and standardization, a sound interpretation of the PCR results can be made only in the context of the combined clinical, laboratory, radiological, and EEG findings. In the absence of indicative clinical parameters, a positive PCR results is highly suggestive of a false positive result which, in the worst case, leads to unjustified continuation of acyclovir treatment. However, a negative PCR result despite all clinical parameters being suggestive for HSE with high probability represents a false negative result. In this case, a second lumbar tap should be considered knowing that the sensitivity of HSV detection drops below 50% after 7 days of acyclovir therapy [Lakeman et al., 1995]. Suboptimal clinical samples which do not fulfill the preanalytical requirements can hardly be recognised when arriving in the laboratory, and bear the inherent risk of yielding false negative results. In addition, PCR analysis of CSF *per se* includes the possibility of false negative results [Lakeman et al., 1995]. This dilemma is also reflected in the diagnostic algorithm for management of patients with suspected HSE as recently presented by a consensus report of the EU Concerted Action on Viral Meningitis [Cinque et al., 1996].

In conclusion, there is a requirement for standardization of all critical aspects of the PCR procedure for HSV. Multicentre comparisons and inter-laboratory quality controls at affordable costs are necessary to establish finally PCR as the "gold standard" that works in the routine laboratory.

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